ClpP is involved in the stress response and degradation of misfolded proteins in Salmonella enterica serovar Typhimurium

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Components of the ATP-dependent Clp protease complex are found in a wide range of prokaryotic cells and they are often expressed as part of the cellular stress response. To investigate the physiological role of the proteolytic subunit, ClpP, in Salmonella enterica serovar Typhimurium (S. typhimurium) an in-frame deletion of the clpP gene was constructed. Growth experiments revealed that clpP is important for the ability of S. typhimurium to grow under various stressful conditions, such as low pH, elevated temperature and high salt concentrations. Since the stationary-phase sigma factor, RpoS, is a target of the Clp proteolytic complex, the effect of the clpP deletion in the absence of RpoS was examined; it was observed that growth of the S. typhimurium clpP mutant is affected in both an RpoS-dependent and an RpoS-independent manner. Analysis of the degradation of abnormal puromycyl-containing polypeptides showed that ClpP participates in the proteolysis of such proteins in S. typhimurium. These findings prompted an investigation of the growth of an Escherichia coli clpP mutant under various stress conditions. The growth of this E. coli mutant was affected by heat, salt and low pH, although not to the same extent as observed for the S. typhimurium clpP mutant. The results of this study indicate that the S. typhimurium clpP mutant is generally more sensitive to environmental stress than the E. coli clpP mutant and it is proposed that this is due to a reduced ability to degrade misfolded proteins generated under these conditions.

Keywords: Salmonella typhimurium, Clp protease, RpoS

INTRODUCTION

The continuous requirement for adaptation of bacteria to starvation and physical stress has forced the development of very complex regulatory networks that respond to changes in the environment. During stress, abnormal or misfolded proteins will accumulate in the cell due to denaturation and errors in biosynthesis. The cell responds to this accumulation by increasing the synthesis both of molecular chaperones, which assist the proper folding or refolding of proteins, and of proteases, which degrade the proteins that cannot be refolded (Goff & Goldberg, 1985). Energy-dependent protein degradation is important in both prokaryotic and eukaryotic cells and it is carried out by multimeric protein complexes, such as the proteasome of eukaryotic and archaeal cells (Kessel et al., 1995) and the ATP-dependent proteases of bacterial cells (De Mot et al., 1999). In addition to eliminating abnormal proteins, proteolysis also controls the level of naturally short-lived regulatory proteins (Mhammedi-Alaoui et al., 1994; Schweder et al., 1996) and therefore is essential for cell homeostasis and optimal metabolic activity (Gottesman, 1996).

In Escherichia coli, several ATP-dependent proteases have been characterized (for reviews see Gottesman, 1996; Miller, 1996; Porankiewicz et al., 1999). Among these is the Clp protease, which together with Lon accounts for up to 80% of the protein degradation in the cell (Goldberg et al., 1994; Laskowska et al., 1996; Porankiewicz et al., 1999). The Clp protease complex consists of a proteolytic component, ClpP, to which substrate specificity is conferred through association with either of the ATPases, ClpA or ClpX. Beside their functions in proteolysis, both ClpA and ClpX possess...
chaperone-like activities (Wawrzynow et al., 1995; Wickner et al., 1994). The Clp protease complex mediates the turnover of specific short-lived regulatory proteins (Mhammedi-Alaoui et al., 1994; Schweder et al., 1996), among them the stationary-phase sigma factor, σ^A (RpoS). RpoS regulates the expression of more than 50 genes in the response to stress or the entry into stationary phase (Hengge-Aronis, 1996, 2000; Loewen & Hengge-Aronis, 1994; Schweder et al., 1996). During exponential growth the level of RpoS is low, in part due to degradation by ClpXP (Lange & Hengge-Aronis, 1994). When cells enter stationary phase or encounter various stress conditions, the concentration of RpoS increases as a result of greater resistance to degradation by ClpXP (Schweder et al., 1996; Webb et al., 1999; Zgurskaya et al., 1997).

Components of the Clp complex are highly conserved in prokaryotic cells (Maurizi et al., 1990a; Wawrzynow et al., 1996). In Gram-positive bacteria ClpP is required for survival under various kinds of stress (Frees & Ingmer, 1999; Msadek et al., 1998) and it has been shown that ClpP participates in the degradation of misfolded proteins generated under these conditions (Frees & Ingmer, 1999; Gaillot et al., 2000; Kruger et al., 2000). In Gram-negative bacteria the role of ClpP during stress is less clear, as indicated by the lack of obvious phenotypes of an E. coli clpP mutant (Maurizi et al., 1990b). In recent studies mutants were generated in the clpP gene of Salmonella enterica serovar Typhimurium (referred to as Salmonella typhimurium) (Hensel et al., 1995; Webb et al., 1999; Yamamoto et al., 2001). S. typhimurium is a facultative intracellular pathogen that upon contact with host cells can promote its own entry (Galan, 1996). In this organism clpP is required for virulence in a mouse assay (Hensel et al., 1995; Webb et al., 1999; Yamamoto et al., 2001) and for growth and survival within peritoneal macrophages (Yamamoto et al., 2001). ClpP has also been found to be involved in the regulation of flagellum biosynthesis in S. typhimurium, and the lack of ClpP leads to a hyper-flagellate cell (Tomoyasu et al., 2002).

Since S. typhimurium encounters various hostile conditions during the infection process (Foster & Spector, 1995), we were prompted to investigate the importance of ClpP for growth in the presence of stress. We find that clpP mutant cells have a reduced ability to grow compared to wild-type cells when exposed to high temperature, low pH or a high salt concentration. Furthermore, we demonstrate that the clpP mutant degrades puromycin-containing polypeptides to a lesser extent than the wild-type, indicating that S. typhimurium ClpP is important for the degradation of misfolded proteins generated when exposed to stress.

**METHODS**

**Media and bacterial strains.** The bacterial strains used in this study are shown in Table 1. Luria–Bertani (LB) broth and M63 medium (Miller, 1992) were prepared as liquid or solid (1.5% agar) media. Unless otherwise stated the strains were grown in LB. Antibiotics were used at the following concentrations for both S. typhimurium and E. coli: 50 µg ampicillin ml⁻¹, 30 µg chloramphenicol ml⁻¹ and 20 µg tetracycline ml⁻¹.

**General methods.** P22 transductions were performed with P22HT105/int 201 as described by Maloy et al. (1996). For plasmid constructions, E. coli DH5α was used. S. typhimurium KP1274 was used for transfer of DNA from E. coli to S. typhimurium. Electroporation and plasmid transformations were performed as described by O’Callaghan & Charbit (1990) and Sambrook et al. (1989). Plasmid purification was performed according to the manufacturer’s instructions (Qiagen).

**Construction of a S. typhimurium clpP deletion mutant.** Using a replacement recombination technique, a recombinant strain of S. typhimurium C5 carrying an 80 amino acid in-frame deletion of clpP was constructed. By PCR amplification of chromosomal S. typhimurium DNA a 750 bp fragment carrying part of the upstream region of clpP was obtained using the primers ClpPC1 (5’-AGTAGATCTCGTCTGTTACGAGATCC-3’) and ClpPC2 (5’-AGAGAATTCGGTTCGCCCATTACAAATGGTGC-3’), while a 642 bp fragment carrying the downstream part of clpP was obtained using the primers ClpPC2 (5’-CTCGAATTCCTGGATTAGAAATGAGG-3’) and ClpPC1 (5’-CTAAGCGTCGCCATGCTGATCGTACG-3’).

The two fragments were digested with EcoRI/BglII and EcoRI/HindIII, respectively, and cloned into the BamHI–HindIII sites of the thermosensitive vector pTS29 (Phillips, 1999), resulting in the plasmid pLT11, carrying a 1376 bp insert.

S. typhimurium C5 was transformed with pLT11 by electroporation and integration was promoted by incubation at 42 °C in the presence of ampicillin. To excise the plasmid from the chromosome, the integrants were grown overnight at 30 °C and plated in the presence of ampicillin. The excised plasmid was cured by incubation of the strain in the absence of antibiotics at 42 °C. Forty ampicillin-sensitive colonies were analysed by PCR to identify mutants with an internal deletion. One colony gave a single 1376 bp fragment, corresponding to the clpP gene with a 240 bp deletion; the wild-type strain C5 gave a single 1616 bp fragment (data not shown). The correct construction of the resulting clpP mutant (LT100) was verified by sequencing the clpP gene.

**Growth experiments.** Growth was followed by diluting cultures (grown overnight at 37 °C in LB) 100-fold into LB and incubating either at 37 °C, at 45 °C, at 37 °C with 5% NaCl, or at 37 °C with the pH reduced to pH 4.5. The optical density was measured at 450 nm (OD₄₅₀).

In plating experiments overnight cultures were diluted 100-fold in LB and allowed to grow to OD₄₅₀ 0.4 at 37 °C. Samples (10 µl) of culture were spotted on plates with or without 5% NaCl. Plates were incubated overnight at either 37 °C or 45 °C.

**Immunoblotting.** Western blot analysis using monoclonal anti-σ^A antibodies (obtained from Neoclon) was performed essentially as described by Lee et al. (1995). Cells were grown to the mid-exponential growth phase (OD₆₅₀ 0.4) or late stationary phase (15 h growth) in LB. Equal amounts (5 µg) of protein were loaded for each sample.

**Two-dimensional protein gel electrophoresis.** Two-dimensional SDS-PAGE analysis was performed as described by Spector et al. (1986) with minor modifications. Strains were grown in M63 supplemented with 0.05% Casamino acids at 37 °C with 5% NaCl.
RESULTS AND DISCUSSION

Measurement of degradation of puromycyl-containing polypeptides. The experiment was performed essentially as described by Raina & Georgopoulos (1990). S. typhimurium wild-type and clpP mutant cells were grown at 37 °C in M63 until the OD<sub>600</sub> reached 0.4. The cells were subsequently incubated with puromycin (200 µg ml<sup>-1</sup>, Sigma) for 10 min and then labelled with 35S-translable (40 µCi ml<sup>-1</sup>, 1.48 x 10<sup>8</sup> Bq µl<sup>-1</sup>; ICN Pharmaceuticals) for 3 min. In the first dimension proteins were separated using ReadyStrip IPG Strips pH 4–7 (Bio-Rad) and in the second dimension an SDS–5% polyacrylamide gel was used.

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RESULTS AND DISCUSSION

ClpP is important for growth under stress conditions in S. typhimurium

With the aim of investigating the physiological role of ClpP in S. typhimurium C5, we constructed a clpP mutant (LT1100) by deleting an internal fragment corresponding to 80 amino acids, including the three amino acids known to be required for the proteolytic activity of ClpP in E. coli (Maurizi et al., 1990a; Wang et al., 1997). By Western-blot analysis using a ClpX antibody we confirmed that the deletion did not affect the expression of ClpX located downstream of clpP (data not shown). When we investigated the growth of LT1100 at 37 °C the growth rate was comparable to that of the wild-type in both enriched (Fig. 1) and minimal broth (data not shown). However, when mutant cells were shifted to 45 °C, the growth was impaired compared to the wild-type, as observed by a reduction in the growth rate and by the inability to reach the same density as the wild-type even after 24 h (Fig. 1). A high salt concentration (5% NaCl) as well as low pH (pH 4.5) also reduced the growth rate of the S. typhimurium clpP mutant (Fig. 1). Furthermore, when mutant and wild-type cells were plated either at 45 °C or in the presence of 5% salt the ability of the mutant to form colonies was greatly reduced (data not shown). To verify that these differences were due to the lack of clpP, we repaired the deletion by transducing the clp<sup>P</sup> allele together with Tn<sub>10</sub> from JF3717 into LT1100, resulting in LT1102 (clp<sup>P</sup>). Under all conditions tested LT1102 grew like wild-type cells (data not shown), confirming that it is the lack of functional ClpP that affects the growth during stress. Thus, our results reveal that the growth of the S. typhimurium clpP mutant is impaired when exposed to stress.

ClpP affects growth of S. typhimurium independently of RpoS

In both S. typhimurium and E. coli, the ClpXP protease is involved in the regulation of the level of RpoS by degradation and a clpP mutation results in increased concentrations of RpoS (Schweder et al., 1996; Webb et al., 1999). Since RpoS regulates or augments the expression of many stress-regulated genes in S. typhimurium and E. coli (Hengge-Aronis, 1996; Ibanez-Ruiz et al., 2000; Loewen & Hengge-Aronis, 1994; O’Neal et al., 1994) we addressed whether the stress sensitivity observed for the clpP mutant was related to rpoS by

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Table 1. Bacterial strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. typhimurium</td>
<td>UK1 rpoS::Ap</td>
<td>Lee et al. (1995)</td>
</tr>
<tr>
<td>JF3717</td>
<td>UK1 xba-6014::Tn10dCm</td>
<td>J. W. Foster, unpublished data</td>
</tr>
<tr>
<td>JF3487</td>
<td>UK1 clpP1::Tn10dTc</td>
<td>Webb et al. (1999)</td>
</tr>
<tr>
<td>C5</td>
<td>Virulent wild-type</td>
<td>Hormacche (1979)</td>
</tr>
<tr>
<td>LT1100</td>
<td>C5 ΔclpP</td>
<td>This work</td>
</tr>
<tr>
<td>LT1102</td>
<td>LT1102 with Tn10 linked to clpP&lt;sup&gt;+&lt;/sup&gt; (linkage 48%)</td>
<td>JF3717 × LT1100, this work</td>
</tr>
<tr>
<td>LT1103</td>
<td>LT1102 clpP1::Tn10dTc</td>
<td>JF3487 × LT1102</td>
</tr>
<tr>
<td>LT1104</td>
<td>LT1100 rpoS::Ap</td>
<td>JF2690 × LT1100, this work</td>
</tr>
<tr>
<td>LT1108</td>
<td>LT1102 rpoS::Ap</td>
<td>JF2690 × LT1102, this work</td>
</tr>
<tr>
<td>LT1115</td>
<td>C5 clpP1::Tn10dTc</td>
<td>JF3487 × C5</td>
</tr>
<tr>
<td>KP1274</td>
<td>LT2 (metA22 metE55 val galE496 rpsL120 xyl-404 H1-b nml H2-e,n,x hslD6 hsdSA29 (r&lt;sup&gt;+&lt;/sup&gt; m&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>Enomoto &amp; Stocker (1974)</td>
</tr>
<tr>
<td>E. coli</td>
<td>F&lt;sup&gt;−&lt;/sup&gt; ΔlacZAM15ΔlacZYA–argFU169 deoR recA1 endA1 hsdR17</td>
<td>Hanahan (1985)</td>
</tr>
<tr>
<td>DH5α</td>
<td>(r&lt;sub&gt;C&lt;/sub&gt; m&lt;sub&gt;i&lt;/sub&gt;) supE44 thi− lpr− lac&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Schultz et al. (1988)</td>
</tr>
<tr>
<td>AMS6</td>
<td>K-12 (r&lt;sup&gt;−&lt;/sup&gt; F&lt;sup&gt;−&lt;/sup&gt; Δlac)</td>
<td>Schweder et al. (1996)</td>
</tr>
<tr>
<td>AMS6P</td>
<td>AMS6 but clpP, Cam&lt;sup&gt;+&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

37 °C until the OD<sub>600</sub> was 0.4. The cultures were then transferred to 45 °C and allowed to grow for 1h. Samples were labelled with 35S-translable (40 µCi ml<sup>-1</sup>, 1.48 x 10<sup>8</sup> Bq µl<sup>-1</sup>; ICN Pharmaceuticals) for 3 min. In the first dimension proteins were separated using ReadyStrip IPG Strips pH 4–7 and in the second dimension an SDS–5% polyacrylamide gel was used.
the absence of ClpP in *E. coli* also affects growth during stress

In *E. coli* the Clp protease degrades intrinsically unfolded protein substrates such as the CRAG protein (Kandror *et al.*, 1999) and a non-secreted alkaline phosphatase mutant protein (Huang *et al.*, 2001), indicating that the proteins formed during stress could be degraded by Clp. The results we obtained with *S. typhimurium* therefore prompted us to analyse how an *E. coli* clpP mutant behaved when exposed to stress using the same experimental conditions as for *S. typhimurium*. In agreement with a previous finding (Maurizi *et al.*, 1999b), we found that growth of the *E. coli* clpP mutant was identical to that of the wild-type at 37 °C. However, when the mutant was shifted to 5% NaCl, to 45 °C or from neutral pH to pH 4.5, we reproducibly obtained results showing that the growth was impaired compared to the wild-type (Fig. 2) although not to the same extent as observed for the
S. typhimurium clpP mutant. Thus, our results indicate that the S. typhimurium clpP mutant is generally more sensitive to stress than the E. coli clpP mutant.

ClpP participates in proteolysis of misfolded protein

In previous studies it has been shown that ClpP is important for degradation of misfolded proteins in the Gram-positive bacteria Bacillus subtilis, Listeria monocytogenes and Lactococcus lactis, whereas degradation of such proteins in E. coli is essentially unaffected by a clpP mutation (Frees & Ingmer, 1999; Gaillot et al., 2000; Kruger et al., 2000; Maurizi et al., 1990b). With the aim of investigating the turnover of misfolded protein in the S. typhimurium clpP mutant, both mutant and wild-type cells were grown in the presence of the tRNA analogue puromycin, which interferes with translation, resulting in the production of misfolded puromycyl-containing peptides. Interestingly, we observed a decrease in colony size of the clpP mutant compared to the wild-type, suggesting that the clpP mutant is more sensitive to puromycin (data not shown). To examine whether a mutation in the clpP gene affects cellular proteolysis in S. typhimurium, the rate of degradation of puromycyl-containing polypeptides in LT1100 and wild-type cells was determined as described by Raina & Georgopoulos (1990). The result, presented in Fig. 3, shows that the clpP mutant degrades these peptides both at a reduced rate and to a lower extent than the wild-type, suggesting that ClpP is involved in the overall degradation of misfolded proteins in S. typhimurium and that the impaired growth of the mutant could be a result of the accumulation of misfolded proteins. In the literature it has furthermore been reported that the phenotype of an E. coli clpP lon double mutant resembles that of a strain carrying a mutation in lon (Maurizi et al., 1990b), the major ATP-dependent protease responsible for degradation of proteins generated during stress in E. coli (Chung & Goldberg, 1981; Maurizi et al., 1985). In contrast, the S. typhimurium clpP lon double mutant grows poorly (Wang et al., 1999) although the phenotype of a S. typhimurium lon mutant is similar to that observed in E. coli (Downs et al., 1986). These observations further support the notion that ClpP has a more prominent role in degrading misfolded proteins accumulating during stress in S. typhimurium than in E. coli.

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Role of S. typhimurium clpP in stress response


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